GROWTH AND LONGEVITY OF XANTHOMONAS MALVACEARUM (E. F. SMITH) DOWSON IN CULTURE

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INTRODUCTION

Very erratic results were obtained by Brinkerhoff when Xanthomonas malvacearum (E. F. Smith) Dowson, the causal organism of bacterial blight of cotton, was cultured on different agar media.[1] Colonies of this organism did not grow on potato-dextrose agar and potato-carrot-dextrose agar when dilutions of bacterial suspensions were used as inoculum. However, growth occurred on both media when mass transfer inoculations were made. It was found that pH and the method of preparing the media were correlated with the growth of this organism. However, no very definite conclusions on this subject were reached.

For the long-term conservation of this organism, this laboratory had tried the method of oil-covered slant preservation. Some cultures when thus stored lost their pathogenicity for some differential varieties of cotton. Moreover, this method had the disadvantage that the tubes must be stored in an upright position at all times, and cultures preserved by this method were susceptible to contamination during preparation and storage. Many methods have been described for the preservation of bacteria by drying. The methods to be described in this study required relatively little specialized equipment and were relatively simple and convenient.

The objectives of this study have been to obtain a better understanding of the growth of X. malvacearum on nutrient agar and nutrient agar

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[1] Unplublished report of L. A. Brinkerhoff.

with glucose, and also to investigate the ability of this organism to survive when dried on small perforated glass beads and in sterile dry soil.

REVIEW OF LITERATURE

Influence of Preparation of Media on Growth of Microorganisms

Data presented by Lewis (33) showed that X. malvacearum failed to grow in liquid culture media containing glucose and various nitrogen compounds when sterilized at 122°C. for fifteen minutes. He attributed the inhibitory effect to "conversion of nitrogen compounds into a form which was not suitable for assimilation by some species of bacteria."

Conversely, Fulmer and Huesselmann (15) reported that heating of sugar solution under pressure with ammonium chloride, dipotassium phosphate or a mixture of the two salts let to the production of stimulative factors for Saccharomyces cerevisiae Hans. They stated that the stimulation was not due to a change in the pH of the media but possibly to the caramelization of the sugar. Fulmer, William, and Werknam (16) extended this work and observed that stimulation was not due to the caramelization of the sugar. They concluded that sterilization of the media under pressure led to the production of growth stimulanting compounds.

The results of Fulmer, William, and Werknam were confirmed by Smiley, Niven, and Sherman (40) and by Niven (35), who also noted that a stimulatory effect was produced if glucose was sterilized in a medium containing phosphate, or sterilized separately with phosphate, ammonium hydroxide or sodium hydroxide. It was suggested that the stimulatory effect was due to the formation of acetaldehyde, pyruvate or similar substances which served as "trigger" compounds by accepting hydrogen in the

initial dehydrogenation of triose phosphate. More recently, Ramesy and Lankford (36) reported that the stimulatory compound(s) had not been identified but did not appear to be an aldehyde or ketone.

Lankford et al. (24), in their study of the effect of sterilizing glucose in culture media on growth of microorganisms, pointed out that autoclaving glucose or other reducing sugars, in certain culture media for the gonococcus, rendered peptone cysteine partially unavailable for growth. He concluded that with glucose sterilized in the medium there was partial inactivation of other essential nutrilite(s), possibly as a result of the combination of the relative functional groups with aldehyde degradation products of glucose.

Hill and Patton (21) found that the slight discoloration occurring during the autoclaving of media (casein hydrolysate) for the microbiological assay for l-tryptophane was caused by an interaction with glucose, which resulted in decreased growth of Streptococcus faecalis R. Their data indicated that decreased growth was due not to formation of growth inhibitors as products of the browning reaction but to an actual destruction of part of the tryptophane (22).

McKeen (34) found that growth of Phytophthora fragariae Hickman was completely inhibited on the media in which 2.0-10.0% dextrose was autoclaved with oatmeal, potato, and lima-bean agars. He attributed the inhibitory effect to toxic or fungistatic substances formed through the interaction of the dextrose with constituents of the media during autoclaving. He also pointed out that the slightly brownish reaction which occurred in these media was similar to the interaction between glucose and amino acids, and was toxic to this organism.

The Effect of Drying on The Survival of Bacteria

Numerous authors have reported survival of microorganisms after drying by various methods, usually in vacuo and often at low temperature. Brown (4,5) reported considerable success in preserving pneumococci and hemolytic streptococci by drying loopfuls of blood or serum suspensions of cells on pieces of filter paper or glass cover slips in bottles containing calcium chloride. These bottles, with covers placed loosely on waxed rims, were evacuated under a bell jar, and sealed by admitting air to the bell jar, thus pressing the loose covers down on the waxed rims. Of forty strains of bacteria dried by this method, all but two remained viable from four to twelve years (6).

Harris and Lange (17) using Brown's technique reported the successful preservation of tubercle bacilli for one year, with the simple modification that they dispensed with the filter paper strips and transferred some of the growth from 4-week-old cultures directly to the inside of glass vials.

Leifson (29) developed a modification of Brown's method, which avoided the entry of air during sealing. He used a rectangular glass jar, with a ground lid rather larger than the top of the jar. This was perforated near one edge, and evacuated through a tube that was sealed in the lid with wax. When it was desired to seal the jar, it was only necessary to slide the lid to one side, so that the hole was brought outside the edge of the jar. The only other modification from Brown's method was that the bacterial suspensions were dried on small perforated glass beads (2-3 mm diameter) as well as on filter paper. He used calcium chloride, and his vacuum was stated to be about 0.01 mm Hg. By this method Salmonella typhi Warren and Scott survived to 64 days.

Stamp (41) described a method in which single drops of thick suspensions were dried slowly on pieces of waxed filter paper at a vacuum of 100 to 300 mm of Hg over P_2O_5 for two to three days at room temperature. The dried disks then were transferred to sterile plugged containers and stored over P_2O_5.

A simple method was described by Rhode (37). A loopful of horse serum was deposited on the inner wall of the small sterilized tube, and a loopful of growth from a culture on a suitable solid medium was emulsified in the serum. This small tube was plugged with cotton and inserted into the wider tube containing P_2O_5 at the bottom. The outer tube was constricted near its upper end and attached directly to a tube connected to a Hyvac pump for exhaustion. The tube was sealed off in vacuo at the constriction and stored in the dark at room temperature. By this method, 83% of cultures representing over forty genera were found to be viable at varying intervals up to 14 years.

Hunt et al. (23) spread a few drops of a broth culture of the organism on porous porcelain beads over silica gel or anhydrous calcium sulfate in tubes. Then the tubes were tightly closed with the cap, and dipped in melted paraffin to provide additional sealing, In their second method, the inoculated beads were first dried in vacuo at room temperature, then placed in small sterile screw-capped vials which were tightly stoppered and slipped into a large screw-capped tube containing the desiccant. All the ten cultures they tested were recovered from beads after 10 months.

There were few citations in the literature concerning storage of bacteria in soil. The principles of these techniques are almost the same as the methods mentioned above. Frobisler et al. (12) devised a method

which was essentially the same as Brown's, except that their bacterial suspensions in sterile rabbit's blood were mixed with sand in the drying tubes. They used a Mason fruit jar and sealed the lid with a ring of plasticine. A small hole about 0.5 mm in diameter was made in the plasticine ring with a needle. When air was admitted suddenly at the end of evacuation, the lid was forced down and the hole closed. By this method they reported that 38 out of 42 strains of beta hemolytic streptococci were alive after 18 years.

Weiss (42) described a method which was somewhat similar to the method used in this study. A 0.05-0.1 ml portion of broth culture or a cell suspension washed from an agar slope was placed with a pipette on 1 ml of sterile dry soil in the tube. The tube was placed in a disiccator until dry or it was tightly stoppered if the infested soil already appeared dry.

MATERIALS AND METHODS

An isolate of race 1 (Texas R-1) of X. malvacearum was used in the study of bacterial growth on nutrient agar with and without glucose. Nutrient agar contained 0.5% peptone, 0.3% beef extract, and 1.5% agar.[2] In preparation of nutrient agar with glucose,[3] the latter ingredient was sterilized with nutrient agar in an autoclave at 122°C. For either fifteen minutes, or one hour. Glucose was also added, aseptically, after passing through fritted-glass filters, or after being sterilized separately in the autoclave, to the hot, melted sterilized nutrient agar. Secondary potassium phosphate (K_2HPO_4)[4] added to nutrient agar with and without glucose was sterilized separately in the autoclave. The pH values of the sterilized glucose solutions, nutrient agar with and without glucose, culture media, and bacterial ooze were determined with a Beckman pH meter. The sterilized media were adjusted to various pH values by use of 0.1 N sterile solutions of sodium hydroxide or hydrochloric acid before they were poured into the plates. One loop (of approximately 2 mm diameter) of a 4-day culture on nutrient agar with dextrose was added to 10 ml of sterile distilled water, shaken and the bacterial suspensions diluted serially. Inoculation was effected by pouring 0.5 ml of the serial dilutions on the surface of the solidified

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[2] A product of Difco Laboratories.

[3] D (+) dextrose, c.a. anhydrous, a product of W. H. Curtin & Co.

[4] A product of J. T. Baker Chemical Co.

media. The bacterial suspensions were spread evenly on the media by means of a large sterile wire loop, and the plates incubated in an air conditioned laboratory with temperatures around 27-29°C.

To test the survival of X. malvacearum on glass beads, the technique developed by Hunt et al. (23) was used with but little modification in the following studies. Five isolates of this organism were used: namely, 61-18, R-1, 62-51, 61-85, and 62-1 (respectively race 10, race 1, race 3, race 9, and race 1).[5] Each isolate was cultivated on three different kinds of media: nutrient agar containing 2% glucose and 0.2 K_2HPO_4, nutrient broth, and nutrient broth with 2% glucose. Small glass beads (as used in handicraft bead-work) were used as carriers of broth or bacterial ooze from agar cultures. They were first sterilized in an oven at 200°C. for 2 hours, and then stored in a desiccator over $CaCl_2$ until used.

For soil storage, 10 grams of dried soil were placed in screw-cap tubes and sterilized with caps off in a dry oven at 200°C. for two hours.

The media used for the physiological tests (Table X) were prepared according to formulas provided by Dr. E. A. Grula of the Microbiology Department, Oklahoma State University; and Dowson (8). The carbohydrates added to the base media for fermentation tests were sterilized separately in the autoclave.

Five differential varieties of cotton were grown for pathogenicity

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[5] Races were based on the system of classification proposed by a committee of the Cotton Disease Council of cotton plant pathologists in the United States. See Proceedings of the 23rd Meeting of the Cotton Disease Council, Jan., 1963.

tests (3). These were CR4, Ac44, 1-10B, 20-3, and BR-1. Inoculations were made when the cotyledons of the seedlings had expanded. Five-ml glass syringes with rubber tips were used to inject inoculum through the stomata or through wounds in the leaves (3).

Fifteen by 150 mm screw-cap tubes were half filled with anhydrous $CaSO_4$, which was covered by a loose glass wool plug. The tubes were sterilized by heating with the cap off, and cooled in a deisiccator. The caps were sterilized separately in an autoclave. The glass beads were heated to incandescence on a wire needle in a gas flame and dipped when cooled into bacterial cultures, and then placed on top of the glass wool in the sterile tubes. The tubes were tightly closed with the screw caps, and then further sealed by dipping the capped end in melted paraffin. In this manner the beads dried rapidly at room temperature. Afterwards the tubes were stored in the freezing compartment of a refrigerator and at room temperature.

In another procedure the inoculated beads in screw-cap perfume bottles were first dried over $CaCl_2$ at room temperature, and then tightly stoppered and sealed with paraffin. Later these were stored in the freezing compartment of a refrigerator at -12°C.

For soil storage, 0.5 ml of a broth culture was placed with a pipette on the sterile dry soil in the tubes, and then dried over $CaCl_2$ with caps loosened. The tubes of infested soil were kept in the freezing compartment of a refrigerator at -12°C. and at room temperature.

EXPERIMENTAL RESULTS

Development and Growth of Colonies of X. Malcacearum on Nutrient Agar with Various Proportions of Glucose

The sugars were added as a dry powder to hot, sterilized nutrient agar. The results showed that nutrient agar with 2%, 4%, 8%, and 16% glucose was favorable for the initiation and growth of this organism (TABLE I). However, no growth appeared on the nutrient agar with 32% glucose (TABLE I). The size of colonies on the nutrient agar with 16% glucose was less than one half the size of those on the nutrient agar containing 2%, 4%, and 8% glucose. The number of colonies on nutrient agar with 8% glucose was significantly less than on the other three media.

Development And Growth of Colonies of X. Malvacearum on Nutrient Agar with Various Amounts of Glucose as Related to Methods of Preparation

Glucose, sterilized by means of fritted-glass filters, was added to nutrient agar. It was also sterilized together with nutrient agar in the autoclave. Colonies developed on nutrient agar with 8%, and 16% filtered glucose (TABLE II). The size of the colonies on the medium with 8% glucose was greater than those on 16% glucose, and the number of colonies per plate was less than for the latter. On the contrary, no colonies emerged on the same media which had glucose sterilized together in the autoclave with the other ingredients. The color of the media to

TABLE I

INITIATION AND GROWTH OF COLONIES OF XANTHOMONAS MALVACEARUM ON NUTRIENT AGAR CONTAINING DIFFERENT AMOUNTS OF GLUCOSE[a]

Medium	Average No. of Colonies Per Plate[b]	Average Size of Colonies in mm
Nutrient agar + 2% glucose	43	5
Nutrient agar + 4% glucose	48	5
Nutrient agar + 8% glucose	22	4
Nutrient agar + 16% glucose	45	2
Nutrient agar + 32% glucose	0	0

[a]Glucose was added as a dry powder to hot, melted nutrient agar, and the surface flooding method of inoculation was used with 0.5 ml of a 4-day nutrient-agar-glucose culture (R-1) diluted 1 x 10^{-5} in sterile distilled water.

[b]The data were taken 4 days after inoculation and are based on 5 replicates. Any two means not underscored by the same line are significantly different at 5% level: 0 22 <u>43 45 48</u>.

TABLE II

INITIATION AND GROWTH OF COLONIES OF XANTHOMONAS MALVACEARUM ON MEDIA AS AFFECTED BY DIFFERENT METHODS OF STERILIZATION[a, b]

Medium	Glucose Added in Relation to Auto-Claving	Average No. of Colonies Per Plate	Average Size of Colonies in mm
Nutrient agar + 8% glucose	after	249[c]	4
Nutrient agar + 16% glucose	after	343[c]	2
Nutrient agar + 32% glucose	after	0	0
Nutrient agar + 8% glucose	before	0	0
Nutrient agar + 16% glucose	before	0	0
Nutrient agar + 32% glucose	before	0	0

[a] The surface flooding method of inoculation was used with 0.5 ml of a 4-day nutrient-agar-glucose culture (R-1) diluted 1×10^{-4} in sterile distilled water.

[b] The data were taken 4 days after inoculation and are averages of 5 replicates of 1 plate each.

[c] Significantly different from each other at the 1% level.

which the glucose solution was added aseptically was different from that of the media in which glucose and the other materials were sterilized together in the autoclave. The color of the latter exhibited a more intense brown color than the former, indicating that a chemical change had taken place during autoclaving. Whatever compound or compounds that were produced or lost inhibited the emergence of colonies of this bacterium on the medium inoculated with a 1×10^{-5} dilution of the bacterial suspension. The pH of the 8% glucose solution by itself dropped from pH 6.8 to 4.4 after autoclaving; and of nutrient agar with 8% glucose autoclaved together, from its initial pH of 6.8 to pH 6.0, while those media with 8% glucose that had been filtered still tested pH 6.8. The pH was not the inhibitory factor for colony initiation on the medium in which nutrient agar and 8% glucose were sterilized together in the autoclave, as the medium was adjusted to pH 7.0 when it was poured.

Growth of X. Malvacearum on Nutrient Agar And Glucose Sterilized Together in The Autoclave

With 8% glucose, the colonies of this organism did not emerge on this medium when inoculated with a 1×10^{-5} dilution of bacterial inoculum (TABLE III), nor with 1×10^{-5} and 1×10^{-4} dilutions (TABLE IV). This bacterium, however, grew luxuriantly on the streaked surface of this medium from a mass of inoculum, as well as from bacterial suspensions of 1×10^{-1}, 1×10^{-2}, and 1×10^{-3} (TABLE IV). This indicates that initiation of colonies of this organism on this medium depends on the concentrations of inoculum used. This organism did emerge on nutrient agar or media in which nutrient agar was added to the nutrient agar with

TABLE III

INITIATION AND GROWTH OF XANTHOMONAS MALYACEARUM ON DIFFERENT PREPARATIONS OF MEDIA[a]

Medium	Average No. of Colonies Per Plate	Average Size of Colonies in mm
Nutrient agar + 8% glucose (autoclaved together) + an equal amount nutrient agar (autoclaved separately)	13	10.0
Nutrient agar + 8% glucose (autoclaved together) + double amount nutrient agar (autoclaved separately)	9	2.0
Nutrient agar	13	2.4
Nutrient agar + 8% glucose (autoclaved together)	0	0

[a]The data are based on 5 replicates. Incubation period was 8 days.

[b]The surface flooding method of inoculation was used with 0.5 ml of a 4-day nutrient-agar-dextrose (2%) culture (R-1) diluted 1×10^{-5} in steril distilled water.

TABLE IV

INITIATION AND GROWTH OF COLONIES OF XANTHOMONAS MALVACEARUM ON NUTRIENT AGAR WITH 8% GLUCOSE AS RELATED TO DIFFERENT METHODS OF STERILIZATION AND CONCENTRATION OF BACTERIAL INOCULUM[a]

Method of Sterilization	Average No. of Colonies Per Plate From Inoculum Diluted[b]					Average Size of Colonies in mm From Inoculum Diluted	
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-4}	10-5
Autoclaved separately	+	+	+	21	4	4.0	4.6
Autoclaved together[c]	+	+	96	0	0		
Autoclaved together[d]	+	+	5	0	0		

[a]The surface flooding method of inoculation was used with 0.5 ml of a 4-day nutrient-agar-2% glucose culture of Race 1. The incubation period was 4 days.

[b]Averages are based on 3 replicates of 1 plate each. The + sign indicates that colonies were too numerous and too crowded to be counted.

[c]Medium stayed in the autoclave for 1 hour after sterilization.

[d]Medium was taken out of autoclave immediately after sterilization.

8% glucose that had been sterilized together in the autoclave (TABLE III). The results of this experiment might be explained on the basis that some essential component of an amino acid, or peptide present in peptone or beef extract reacted with some of the glucose when nutrient agar and glucose were autoclaved together, rendering the medium unfavorable for the growth of single cells of this organism. The essential component was available, however, when nutrient agar or broth that was sterilized separately was later added. Since sufficient of the compound appeared to be transferred with the more concentrated inoculum it would appear that only a small amount is needed, and this for the initiation of growth.

With 2% glucose, the initiation of colonies sometimes occurred regardless of the time the medium stayed in the autoclave (TABLE V). Inoculum diluted 10^{-4} and 10^{-5} which did not grow on the nutrient agar in which 8% glucose had been added before sterilization in the autoclave was able to grow on this medium. The numbers of colonies per plate, however, were much decreased in comparison with that on nutrient agar with 2% glucose autoclaved separately.

Initiation and Growth of Colonies of X. Malvacearum as Related to pH

Nutrient agar and nutrient agar with 2% glucose autoclaved separately were used in this study. The inoculum for both media was the same. The data from this investigation showed that the pH range for this organism to initiate growth on the medium without glucose was 6.8 to 7.6; on the medium with glucose was 6.6 to 8.5 (TABLE VI). Colonies on the latter medium appeared more numerous and bigger than those on the former. The pH of the media tended to decrease with time (TABLE VII). One week after incubation noninoculated nutrient agar had a pH value of 6.5, whereas the

TABLE V

INITIATION AND GROWTH OF COLONIES OF XANTHOMONAS MALVACEARUM ON NUTRIENT AGAR WITH 2% GLUCOSE AS RELATED TO DIFFERENT METHODS OF STERILIZATION AND CONCENTRATION OF BACTERIA INOCULUM[a, b]

Method of Sterilization	Average No. of Colonies Per Plate From Inoculum Diluted[c]					Average Size of Colonies in mm From Inoculum Diluted	
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-4}	10^{-5}
Autoclaved separately (15 min.)	+	+	+	+	22		5
Autoclaved separately (1 hour)	+	+	+	+	20		5
Autoclaved together (15 min.)	+	+	+	189	1	3	3
Autoclaved together (1 hour)	+	+	+	200	3	3	3
Autoclaved together (1 hour)[d]	+	+	+	42	2	3	3
Nutrient agar	+	+	+	+	24		1

[a] The surface flooding method of inoculation was used with 0.5 ml of a 4-day nutrient-agar-glucose culture diluted 1 x 10^{-5} in sterile distilled water. The culture was Race 1. Incubation period was 4 days.

[b] Averages are based on 3 replicates of 1 plate each.

[c] The + sign indicates that colonies were too numerous and too crowded to be counted.

[d] Medium was left in the closed autoclave for an additional hour after the steam was turned off.

TABLE VI

INITIATION AND GROWTH OF COLONIES OF *XANTHOMONAS MALVACEARUM* AS RELATED TO pH[a,b]

pH Immediately After Pouring	Nutrient Agar + 2% Glucose[c]			Nutrient Agar		
	Average No. of Colonies Per Plate	pH After 4 Days	Average Size of Colonies in mm	Average No. of Colonies Per Plate	pH After 9 Days	Average Size of Colonies in mm
5.0	0		0	0		0
5.5	0		0	0		0
6.0	0		0	0		0
6.5	0		0	0		0
6.6	27	6.3	3.5	0	6.5	0
6.8	61	7.0	4.3	6	6.6	1.5
7.0	59	7.0	5.0	8	6.7	1.7
7.2	48	7.1	4.7	2	6.8	2.5
7.4	18	7.2	6.7	3	6.7	1.5
7.6	17	7.4	5.0	4	7.1	2.5
7.8	47	7.7	5.5	0	7.1	0
8.0	65	8.0	5.5	0	7.3	0
8.3	21	7.8	5.0	0	7.9	0
8.4	5	7.9	4.5	0	7.9 [d]	0
8.5	2	8.0	4.0	0	8.1	0

[a] The data are based on 5 replicates.

[b] The surface flooding method of inoculation was used with 0.5 ml of a 4-day nutrient agar-glucose culture (R-1) diluted 1 x 10^{-5} in sterile distilled water.

[c] Glucose was sterilized separately in autoclave.

[d] pH values after 4 days.

TABLE VII

CHANGES OF pH VALUES OF INOCULATED AND NONINOCULATED MEDIA

Medium[a]	Treatment	pH After[b]				
		Pouring	2 Days	4 Days	7 Days	9 Days
Nutrient agar + 2% glucose	0.5 ml 10^{-5} suspension of X. malvacearum, pH 7.0	7.1	6.8	6.8	7.2	7.3
Nutrient agar + 2% glucose	0.5 ml sterile distilled water, pH 7.0	7.1	6.8	6.6	6.6	6.6
Nutrient agar + 2% glucose	none	7.1	6.8	6.6	6.2	
Nutrient agar	0.5 ml 10^{-5} suspension of X. malvacearum	7.0	6.9	6.6	6.6	7.0
Nutrient agar	0.5 ml sterile distilled water	7.0	6.9	6.6	6.4	6.4
Nutrient agar	none	7.0	6.9	6.6	6.5	

[a] Glucose was sterilized separately in the autoclave.

[b] Colonies developed after 2 days on nutrient-glucose agar that was inoculated, and after 4 days on nutrient agar; colonies averaged 13 and 30 in number and were 1 and 5 mm in diameter, respectively.

original pH after pouring was 7.0; nutrient agar with 2% glucose changed from 7.1 to 6.6 when sterile distilled water was used, and to 6.2 when nothing was added. When the colonies of this bacterium started to develop, the pH value of the media began to increase. The drop in pH of the media before growth became evident was apparently a characteristic of the media, as shown by a similar drop in pH of uninoculated plates. The pH readings for these tests indicated that the culture did not produce much acid on nutrient agar with 2% glucose during a short period of incubation (TABLE VI, TABLE VII). However, other tests have shown that the amount of acid produced was related to the concentration of glucose in the medium.[6]

Growth of Streak Transfers of X. Malvacearum and Changes in pH of Media with Different Amounts of K_2HPO_4

A mass culture was streaked on the surface of nutrient agar with 2% glucose that had been sterilized separately in the autoclave, and on nutrient agar. Various amounts of K_2HOP_4 added to both media were sterilized separately in the autoclave. Growth did not occur on the media to which 8% and 16% phosphate were added (TABLE VIII). In the presence of sugar the pH values of the media dropped from their initial pH values to nearly neutral in reaction. Afterward the pH values of the media progressively increased with time (TABLE VIII). In the absence of sugar, on the contrary, the nutrient agar still maintained its initial pH for one week. Afterward the pH values increased progressively with time to

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[6] See footnote [1]

TABLE VIII

RELATIVE GROWTH OF STREAK TRANSFERS OF XANTHOMONAS MALVACEARUM AND CHANGES IN pH OF MEDIA[a]

Medium[b]	pH After Pouring	Growth After 7 Days[c]	pH After[d] 7 Days	18 Days	24 Days
2% glucose + NA + 16% phosphate	8.7	-			
2% glucose + NA + 8% phosphate	8.4	-			
2% glucose + NA + 4% phosphate	8.2	+	7.0	7.5	7.9
2% glucose + NA + 2% phosphate	8.0	++	7.0	7.5	7.5
2% glucose + NA + 1% phosphate	7.8	+++	7.1	7.3	7.3
2% glucose + NA	7.0	++++	6.9	7.9	7.9
NA + 16% phosphate	8.7	-			
NA + 8% phosphate	8.4	-			
NA + 4% phosphate	8.2	+	8.2	8.2	8.2
NA + 2% phosphate	8.0	++	8.0	8.3	8.5
NA + 1% phosphate	7.8	++	8.3	8.3	8.5
NA	7.0	++	7.3	8.3	8.4

[a] Plates were inoculated by streaking with relatively large masses of actively growing cells.

[b] Glucose, NA, and phosphate were autoclaved separately.

[c] -, no growth; +, a little growth but not continuous along streaks; ++, more growth but not continuous along streaks; +++, growth continuous along streaks and fairly abundant; ++++, growth continuous along streaks and abundant.

[d] pH values were measured on the bacterial ooze.

more than pH 8.2 (TABLE VIII). Using Hinsberg's technique (10) the basic compounds produced by this bacterium were identified as secondary amines. Apparently this organism had attacked the amino acids, and broken down the carboxyl group through decarboxylation. Since peptone is not a distinct chemical entity, it is not known from which fraction the metabolic product arose.

Survival of X. Malvacearum after Drying under Various Conditions

To test the survival, one or two inoculated beads or small portions of infested soil were added to nutrient broth containing 2% glucose. Growth was indicated by turbidity of the broth. After 11 weeks of storage on glass beads under all conditions, this organism still retained viability (TABLE IX). This bacterium, however, did not survive in the soil after one month in these tests.

Physiological Tests of X. Malvacearum after Storage

Two isolates, R-1 (race 1), and 61-18 (race 10), were tested for physiological reactions after 7 weeks preservation at room temperature and in the freezing compartment of a refrigerator. Check cultures that had been maintained at room temperature by transfering to new media at about 5-day intervals were used for comparison. The physiological differences between the preserved and check cultures of isolate R-1 were in the fermentation of xylose, reactions in litmus milk, and reduction of nitrate to ammonia (TABLE X). As far as isolate 61-18 was concerned, differences between the preserved and check cultures were detected in the reactions of glucose, glycerol, and gelatinase production (TABLE X). Additional studies need to be made to determine the significance of these

TABLE IX

SURVIVAL OF 5 ISOLATES OF XANTHOMONAS MALVACEARUM ON GLASS BEADS AND IN SOIL

Carrier and Medium on Which Isolates Were Grown[a, b]	Storage Condition	Length of Storage in Weeks 4	7	11
Beads				
NA + 2% glucose + K_2HPO_4	27-29°C	+	+	+
NA + 2% glucose + K_2HPO_4	-12°C	+	+	+
NA + 2% glucose	27-29°C	+	+	+
Soil				
NB	27-29°C	-	-	-
NB	-12°C	-	-	-
NB + 2% glucose	27-29°C	-	-	-
NB + 2% glucose	-12°C	-	-	-

[a]The five isolates were 61-85, 61-18, R-1, 62-51, and 62-1.

[b]NA, nutrient agar; NB, nutrient broth.

TABLE X

PHYSIOLOGICAL REACTIONS OF *XANTHOMONAS MALVACEARUM* AFTER STORAGE ON GLASS BEADS FOR 7 WEEKS COMPARED TO CULTURES CONTINUOUSLY TRANSFERRED

	Cultures and Method of Storage						
	R-1			61-18		Reference	
Test	27-29°C	-12°C	ck	27-29°C	ck	Bergey's (2)	Elliot's (9)
Acid from sucrose	+	+	+	+	+	+	+
Acid from galactose	+	+	+	+	+	+	+
Acid from xylose	-	+	+	+	+	+	+
Acid from glucose	+	+	+	-	+	+	+
Acid from arabinose	-	-	-	-	-	-	-
Acid from maltose[a]	-	-	-	-	-	+	+
Acid from lactose	-	-	-	-	-	+	+
NO_3 reduction to NO_2	-	-	-	-	-	-	-
NO_3 reduction to NH_3	-	+	-	+	+		
Pectate liquifaction	-	-	-	-	-	-	
Lipolytic acion	-	-	-	-	-	-	-
H_2S produced	+	+	+	+	+	+	+
Gelatin liquified	+	+	+	-	+	+	+
Indole formed	-	-	-	-	-		-
Litmus milk	Coagulated	-	Coagulated	Alkaline, rennin curd formed		Casein ppt., and digested	Coagulated and peptonization
Starch hydrolyzed	+	+	+	+	+	+	+

[a] *X. malvacearum* fermented filtrate maltose but did not at all or only slightly fermented the heated maltose (7).

differences.

Pathogenicity Tests with Surviving Cultures

After survival of this organism on glass beads for seven weeks, pathogenicity tests were made on cotton seedlings. Disease readings were taken 2 weeks after inoculation. Preserved cultures stored under all conditions still retained varulence for their respective hosts (TABLE XI). Stored cultures of isolate 62-51 under both storage conditions showed virulence on BR-1, which proved resistant to this culture maintained on media. R-1 isolate preserved in the freezing compartment of the refrigerator exhibited pathogenicity on 1-10B, which was not susceptible to this isolate maintained on media. The erratic reactions of the check cultures of isolate 61-51, and of 61-18 may have been due to mutations that occurred in culture. These tests need to be repeated to determine whether the differences were real.

TABLE XI

PATHOGENICITY OF STORED CULTURES OF XANTHOMONAS MALVACEARUM ON DIFFERENTIAL VARIETIES OF COTTON SEEDLINGS

Isolates and Method of Storage	Blight Reactions of Differential Strains of Cotton[a]				
	Ac44	1-10B	CR4	BR-1	20-3
62-51					
Beads (27-29°C)	+	+	-	+	-
Beads (-12°C)	+	+	-	+	-
Check-on medium	-	+	-	-	-
61-85					
Beads (27-29°C)	+	-	-	+	+
Beads (-12°C)	+	-	-	+	+
Check-on medium	+	-	-	+	+
R-1					
Beads (27-29°C)	+	-	-	-	
Beads (-12°C)	+	+	-	-	
Check-on medium	+	-	-	-	
61-18					
Beads (27-29°C)	+	+	+	+	+
Beads (-12°C)	+	+	+	+	+
Check-on medium		-	-		-

[a] +, susceptible; -, immune or resistant.

DISCUSSION

The results of the foregoing investigation indicated that nutrient agar with glucose enhanced the initiation and growth of colonies of X. malvacearum, provided that the glucose had been sterilized separately, and the medium adjusted to approximately pH 7.0. Inhibition or a decrease of colony initiation and growth occurred on media in which glucose and nutrient agar had been sterilized together in the autoclave, but in these tests lack of growth was not correlated with the pH of the media as the media had been adjusted to pH 7.0 when prepared. The failure of colonies to grow from dilute inoculum (presumably single cells) on nutrient agar containing 8% glucose which had been prepared by autoclaving the ingredients together was overcome when additional nutrient agar was added to this medium. Growth in the combined medium indicates that the browning compounds produced from glucose and nutrient agar during autoclaving were not toxic but the loss or partial inactivation of an essential nutrient(s) was responsible for the lack of growth. Lewin (30,31,32), and Lea and Hanann (25) stated that disappearance of free amino acids and peptides was due to the interaction of amino acids, peptides and glucose. Also Friedman and Kline (11) found that a loss of nutritive value which resulted from the interaction between glucose and amino acids was accompanied by the occurrence of a browning reaction.

Further support for the hypothesis that a toxic material was not produced by autoclaving was the fact that growth occurred from

concentrated inoculum. An enzyme possibly was present in sufficient quantity in the concentrated inoculum to break down the amino acid-glucose complex, however, another explanation for the dilution effect would be that the concentrated inoculum contained necessary vitamins or other growth factors.

According to Lewin the interaction between amino acids and glucose is as follows:

Amino acid + glucose amino acid-N-glucoside

To test Lewin's hypothesis, tryptophane, arginine, and glycine each were autoclaved with glucose. Browning compounds were produced. From this example, it appears that some amino acids and peptides will be altered or inactivated when nutrient agar and glucose are autoclaved together. A preliminary test indicated that colonies were not initiated when tryptophane was added to autoclaved nutrient-glucose agar.

For better initiation and growth of colonies of X. malvacearum on artificial or synthetic media, the results of this investigation have shown that carbon compounds such as glucose should be sterilized separately, and that sterilized media should be adjusted to a suitable pH value by use of a sterilized solution of an alkali such as sodium hydroxide.

The pH of the medium after growth is undoubtedly important in the length of time X. malvacearum grows and survives on a solid medium. The progressive increase of pH on nutrient agar with and without 2% glucose was probably due to the production of secondary amines. Hence, without periodic transfer, alkalinity would be accumulated unless there was sufficient acid produced to neutralize the alkali. Brinkerhoff's data showed that with 4% glucose the medium became acid, and with

potato-dextrose agar which contained much more utilizable carbohydrates the medium became even more acid.[7]

All isolates of this organism in this study survived when dried on glass beads over anhydrous calcium sulfate and calcium chloride for 11 weeks. A possible mechanism of survival was recently suggested by Hawker et al. (20). They stated that freeze-drying, a method widely used to preserve microorganisms, induced hypobiosis--a state of reduced metabolic activity, and that in a state of hypobiosis, microorganisms are able to survive for long periods in the absence of nutrients.

X. malvacearum failed to survive in sterile soil either at room temperature (27-29°C.) or in the freezing compartment of a refrigerator at -12°C. after one month. Russell (38), in her study on X. malvacearum, found that at 30°C. the survival of this organism in nonsterile soil was no longer than 16 days, but survival was much longer at lower temperatures in sterile soil. Of significance to this study may be the fact that Russell found that as the soil moisture was increased the bacterium survived for shorter periods. Many authors agree that rapid removal of water during drying is essential to successful preservation (13,14,39, 41). In this investigation, as considerably more water was added with the inoculum than Russell used, the infected soil probably did not dry out as rapidly as in her tests. Slower drying may have been the reason for the lack of survival in the soil.

More tests are needed with stored cultures of X. malvacearum to determine whether the variations that were observed in physiological characters and in pathogenicity were due to mutation. Brinkerhoff (3)

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[7] See footnote 1.

found that mutations for pathogenicity and resistance to antibiotics could be readily isolated from cultures of this bacterium. Hartsell (18) reported that four species of Bacillus preserved under paraffin oil failed to ferment sucrose. Even Escherichia coli (Migula) Castellani and Chalmers when thus stored lost the capacity to produce indole from tryptophane (19). It was found that variants occurred in Brucella species when stored in the absence of nutrients and when lyophilized (1). Lederberg (26,27,28) also has reported that mutation may lead to the loss of or alternation of enzymatic action in E. coli.

SUMMARY

The growth and cultural reactions of isolate R-1 (race 1) of X. malvacearum were investigated. The survival of isolates R-1, 61-18, 62-1, 61-85, and 62-51 (respectively race 1, race 10, race 1, race 3, and race 9) on small glass beads over anhydrous $CaSO_4$ and in sterile soil was studied. Pathogenicity and physiological reactions of the preserved cultures were also made in this study.

Colonies were initiated from dilute inoculum and grew on nutrient agar with 2%, 4%, 8%, and 16% glucose when the glucose was added aseptically or autoclaved separately, and the medium was adjusted to approximately pH 7.0. No growth of this organism appeared on nutrient agar with 32% glucose, whether the latter ingredient was sterilized separately or autoclaved with the nutrient agar.

Dilutions of 10^{-5} and 10^{-4} bacterial suspensions did not grow when nutrient agar and 8% glucose were sterilized together in the autoclave, but the failure was overcome by adding additional peptone and beef extract after autoclaving. This indicates that autoclaving destroys a required nutrilite rather than producing a toxin, although the production of a competitive inhibitor is another possible explanation.

When the pH of the media was varied, it was found that the bacterium initiated growth between pH 6.8 and 7.6 on nutrient agar withoug glucose and between pH 6.6 and 8.5 on nutrient agar with glucose.

Five isolates of this bacterium that were dried on glass beads

over anhydrous $CaSO_4$ survived for 11 weeks in the freezing compartment of a refrigerator (-12°C) and at room temperature (27-29°C). However, they did not survive in sterile dry soil in test tubes when 0.5 ml of nutrient broth cultures were added and dried over $CaCl_2$ in a desiccator.

The physiological differences of preserved and check clutures of isolate R-1 involved the fermentation of xylose, reactions in litmus milk, and reduction of nitrate to ammonia. The differences in the isolate 61-18 were concerned with the reaction of glucose, glycerol, and gelatinase production.

Preserved cultures stores in the freezing compartment of a refrigerator (-12°C) and at room temperature (27-29°C) retained virulence for their respective hosts. Stored cultures of isolated 62-51 in both conditions become somewhat pathogenic to BR-1. The R-1 isolate that was preserved at -12°C became somewhat pathogenic to 1-10B.

Additional studies are needed to determine whether these differences in physiology and pathology between preserved and check cultures were due to genetic changes in the cultures or to other factors.

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